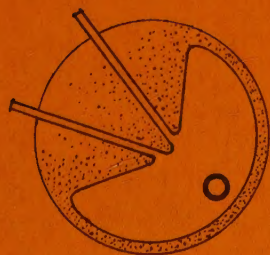


LEIF SESTOFT

CONTROL OF FRUCTOSE
METABOLISM IN
THE PERFUSED LIVER



ODENSE UNIVERSITY PRESS · 1975

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**CONTROL OF FRUCTOSE
METABOLISM IN
THE PERFUSED LIVER**

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Odense Universitet, 18. januar 1975.

Annelise Bach,
h.a. dec.



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PREFACE

It was unexpected to me to experience, as a bachelor of medicine, how close to ignorance we still were in the biological sciences. The acquaintance with D. Benveniste, M.D., prompted me to a personal engagement in scientific research.

After some years with clinical education and fortuitous scientific activities I had the opportunity to establish a collaboration with S.E. Damgaard, M.Sc., and F. Lundquist, Ph.D., which continued during my scholarship at Odense University in the period 1971-1973. During these years the excellent working conditions at the Department of Biochemistry A, University of Copenhagen, was favoured by extensive good-will from the staff of the Institute of Biochemistry, University of Odense, and by grants from several funds, including the Novo foundation, P. Carl Petersens Fond, F.L. Smidth & Co's Jubilæumsfond, and, not least, from the Danish Medical Research Council.

Discussions with N. Grunnet, M.Sc., and H. Thieden, M.D., intensified our interest in metabolic interactions with ethanol, and both groups are still working with these problems employing different techniques.

Hand in hand with the collaborators of N. Tygstrup, M.D., and K. Winkler, M.D., we have participated in the development of pig liver perfusion technique. The opportunity was given to us to be the first to perform well controlled metabolic experiments with this preparation, which since that has proved so valuable for metabolic investigations requiring large amounts of biopsy material.

At the time when we were aware of the problems involved

in describing the elimination of substrates by the liver in pertinent enzyme kinetic terms, P. Fleron appeared as an attendant to the biochemical lessons given by H. Flodgaard, M.D., thus confirming that in the development of science nothing is accidental: when the matter is ripe, the persons are present.

Competent technical assistance was given by Lilian Lund Hansen, Lissi Immerdal and Anita Sommerfeldt. Regarding the pig liver perfusion experiments the technical staffs from the Department of Surgery C, Department of Medicine A (Division of Hepatology) Rigshospitalet and the Department of Clinical Physiology, Kommunehospitalet, Copenhagen, rendered extensive and skillful assistance.

The typewriting was done carefully by Aase Reffstrup and Gydde Phil, secretaries of the Institute of Biochemistry, Odense.

To all people, funds and departments who have helped in some way or another, I devote my best thanks.

Copenhagen, November 1974

Previously published papers:

- a. Thieden, H.I.D., Damgaard, S.E., Grunnet, N. & Sestoft, L.: Effect of fructose and glyceraldehyde on ethanol metabolism in human and rat liver. *Eur. J. Biochem.* 1972:30:250-261.
- b. Sestoft, L., Tønnesen, K., Hansen, F.V. & Damgaard, S.E.: Fructose and D-glyceraldehyde metabolism in the isolated perfused pig liver. *Eur. J. Biochem.* 1972:30:542-552.
- c. Damgaard, S.E., Lundquist, F., Tønnesen, K., Hansen, F.V. & Sestoft, L.: Metabolism of ethanol and fructose in the isolated perfused pig liver. *Eur. J. Biochem.* 1973:33:87-97.
- d. Sestoft, L.: Regulatory processes in rat liver induced by sudden changes in fructose concentration, in Regulation of hepatic metabolism. Alfred Benzon Symposium VI. (Lundquist, F. & Tygstrup, N., eds.) pp. 285-299, 1974. Munksgaard, Copenhagen.
- e. Lundquist, F., Sestoft, L., Fleron, P. & Damgaard, S.E.: Pathways of fructose carbon in the perfused pig liver, in regulation of hepatic metabolism. Alfred Benzon Symposium VI. (Lundquist, F. & Tygstrup, N., eds.) pp. 302-312, 1974. Munksgaard, Copenhagen.
- f. Sestoft, L.: Regulation of fructose metabolism in the perfused rat liver. Interrelation with orthophosphate, glucose, ketone body, and ethanol metabolism. *Biochim. Biophys. Acta* 1974:343:1-16.
- g. Sestoft, L. & Fleron, P.: Determination of the kinetic constants of fructose transport and phosphorylation in perfused rat liver. *Biochim. Biophys. Acta* 1974:345:27-38.

INTRODUCTION

Fructose is supplied to mammals in the food as a part of sucrose or as the monohexose itself. It is absorbed by the small intestine at a slower rate than is glucose (Cori 1926, Holdsworth & Dawson 1964). In the portal vein the fructose concentration may rise up to about 5 mM in the water phase (Hill et al. 1954, Topping & Mayes 1971, Sestoft & Fleron 1974b) however, a minor part of the fructose absorbed is metabolized to glucose or lactate in the intestinal wall (Kiyasu & Chaikoff 1957, Ockerman & Lundborg 1965). In the intestine, in the liver and in the kidney, fructose is metabolized via a specialized enzyme, the ketohexokinase, which has a low K_m with fructose and a high activity (Hers 1952, Adelman et al. 1967, Heinz et al. 1968, Woods et al. 1970, Weiser et al. 1971). This enzyme phosphorylates fructose in the carbon 1-position (Slein et al. 1950, Adelman et al. 1967) in contrast to the hexokinases which phosphorylates fructose in the 6-position. The hexokinases are present also in brain, muscle and fat tissue and has a higher K_m with fructose and low activity at physiological concentrations (Walker 1965). In the liver, kidney and duodeno-jejunal part of the small intestine both types of enzymes are present, but the metabolism of fructose via the ketohexokinase is under normal conditions by far the most important. In the eviscerated animal the concentration of intravenously administered fructose is constant for hours (Bollman & Mann 1931, Wick et al. 1953), and among the fructose metabolizing organs the liver accounts for nearly the total

fructose metabolism (Reinecke 1944, Wick et al. 1953). The investigations presented here have been restricted to the metabolism of fructose by the liver.

Briefly the historical development of the knowledge about fructose metabolism may be subdivided into a period before 1942 in which the rapidity of fructose metabolism and the main fructose metabolizing organs were discovered (cf. a review by Deuel 1936). The discovery of phosphorylation of fructose in the carbon 1-position (Kjerulf-Jensen 1942) initiated a new area which lasted until about 1967, during which period the main pathways of fructose metabolism were elucidated and some of the main enzymes of fructose metabolism were characterized (cf. a review by Leuthardt & Stuhlfauth 1960). In the last decade the knowledge of the particular metabolic consequences of fructose administration was initiated by the discovery of a severe depletion of adenine nucleotides induced by fructose (Perheentupa & Raivio, 1967, Raivio et al. 1969). Since then the dynamic biochemistry of fructose in liver tissue has been very actively investigated. One reason for this is the revival of the liver perfusion technique which has taken place in the same period, another is that the introduction of specific enzymatic assays allowed a much more extensive and detailed description of metabolic interactions and regulations in the intact organ. As regards fructose metabolism much of the recent information has been published in *Clinical and Metabolic Aspects of Fructose Metabolism*, Helsinki, 1972.

METHODS OF INVESTIGATION

The aim of the investigations presented has been to disclose regulatory features of the carbohydrate metabolism in the intact organ, to test regulatory properties predicted from investigations on less complicated systems and to identify regulatory mechanisms under near physiological conditions. The term near physiological conditions does not allude to the fructose concentration in the blood observed under physiological conditions which is low. On the contrary, the investigations have been performed mainly with unphysiologically high fructose concentrations in order to expose the liver to significant metabolic alterations.

The following goals have been persued: a) To obtain a description of the dependence of metabolic alterations on the concentration of fructose under steady state conditions. The definition of steady state was based on the rate of phosphate uptake as this is increased in the initial period after addition of fructose to the system due to accumulation of phosphorylated intermediates (Sestoft 1974 a). Steady state is therefore defined as established when net phosphate uptake has terminated. b) To describe the time dependence of the metabolic changes induced by fructose. To achieve this, perfusions of very short duration were combined with a rapid sampling technique. c) To describe the metabolism of fructose 1-phosphate, perfusions were interrupted when fructose-1-phosphate had accumulated, and the metabolism in the following period was then investigated. d) To investigate the

metabolic interaction between fructose and glucose, ethanol and inorganic phosphate, these substrates were added to the perfusion medium, and further the metabolism of D-glyceraldehyde, a metabolite which is unique for fructose metabolism, was investigated separately. e) Kinetic properties of certain enzymatic reactions were investigated on partially purified preparations or on high speed supernatants in order to identify some regulatory properties observed in the intact organ. f) At present, investigations on the metabolic pathways of fructose are studied by means of fructose which is uniformly or specifically labelled with ^{14}C : To calculate the flux of carbon through the different alternative metabolic pathways a computer simulation of a model system for fructose metabolism has been employed. g) To separate the kinetics of fructose transport across the liver cell membrane from the kinetics of the intracellular phosphorylation in the intact organ, a model for the elimination of fructose was proposed and used for the calculations.

Intact tissue preparations present technical and theoretical problems in testing theories of metabolic control, but on the other hand such preparations also may be valuable in disclosing unknown regulatory properties of enzymatic or transport processes. It should be emphasised that extrapolation of evidence from in vitro to intact systems as well as in the opposite direction is always somewhat speculative.

TECHNIQUES EMPLOYED:

The pig liver perfusion system has been described in details previously (Tygstrup et al. 1971, Sestoft et al. 1972) and so has the rat liver perfusion system (Sestoft 1974 b). In Table I the main differences in the perfusion system have been outlined. Non-recirculating perfusion systems in rat liver and in pig liver when radioactive material was employed was introduced in order to achieve steady state conditions i.e. a well defined constant composition of the affluent medium.

The use of liver slices was given up because of obvious shortcomings of this preparation as for instance a low rate of ethanol oxidation and of oxygen uptake (Thieden et al. 1972) of fructose uptake and lack of ability to synthesize glycogen (Stewart & Thompson 1941, Renold et al. 1954, Krebs et al. 1966) and leakage of metabolites, cofactors and enzymes (Gaja et al. 1968). Only in the case of studies on human liver the slice technique had to be employed (Thieden et al. 1972).

PATHWAYS OF FRUCTOSE METABOLISM IN THE LIVER.

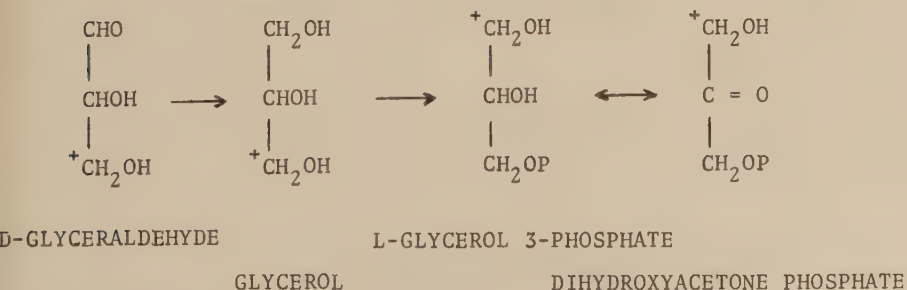
The demonstration that fructose is phosphorylated in the carbon 1-position by the liver (Kjerulf-Jensen 1942) initiated a series of investigations on the metabolic pathways of fructose, which were obviously different from those of glucose. The metabolic scheme for the degradation of fructose 1-phosphate by the liver was suggested by Leuthardt et al.

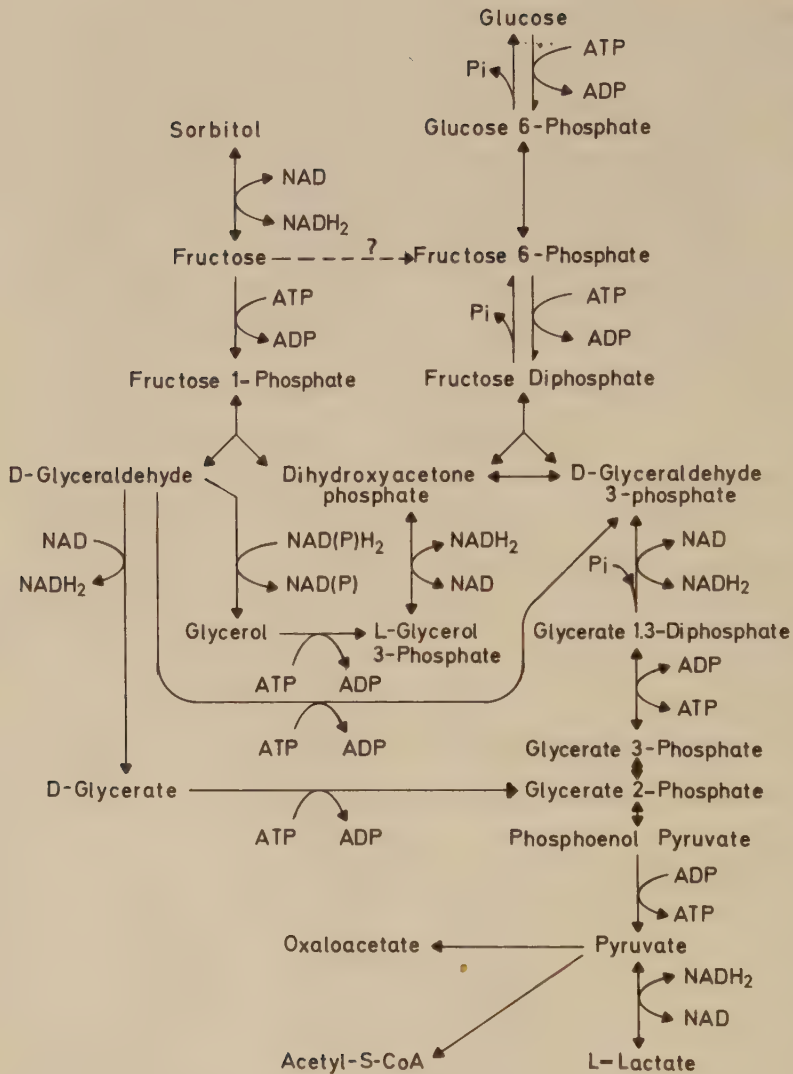
Table 1. Liver perfusion techniques employed. All livers were perfused at 38°.

SPECIES	CIRCULATION & MEDIUM	OXYGENATION	OPERATIVE PROCEDURE	BIOPSIES	REFERENCE
Fed pigs	Recirculating system with 3.5 to 4.0 l fresh pig blood diluted with saline Hematocrit about 20%. pH kept constant by automatic titration. Flow (arterial + portal) about 1 ml/min per g liver.	O ₂ 95% CO ₂ 5%	Liver cooled to 30°C and removed from the animal. Anoxic period less than 3 min.	Biopsy at the end of the experiment.	A
Pigs fasted for 48 h	Recirculating system until addition of ¹⁴ C labelled fructose. Then non-recirculating. Medium as above, but increased to 6 l. Flow about 1 ml/min per g liver.	O ₂ 20% CO ₂ 5% N ₂ 75%	do	Biopsies performed at 2 and 4 min after addition of ¹⁴ C fructose.	B
Rats, fed or fasted for 48 h.	Non-recirculating system. Washed bovine erythrocytes in Krebs Ringer bicarbonate, added 2% bovine albumin. Hematocrit 28%. Flow 0.7 ml/min per g liver.	do	No cooling. Liver left in situ. No anoxic period.	Biopsies performed at the end of the experiment. In some cases one liver lobe was removed at 4.25 min after addition of fructose, and the flow was then reduced proportionally maintaining an unchanged perfusion pressure and an unchanged flow (0.7 ml/min per g liver).	C + D

A: Sestoft et al. 1972, B: Lundquist et al. 1974, C: Sestoft 1974 a, D: Sestoft 1974 b.

1953 and Hers & Kusaka 1953, and in the following years the enzymatic reactions involved in the metabolism of D-glyceraldehyde were investigated separately in more detail (Wolf & Leuthardt 1953, Holzer et al. 1955, Ichihara & Greenberg 1957, Moore 1959, Holldorf et al. 1959). The quantitative importance of the different pathways (outlined in Fig. 1) was investigated in intact animals by means of fructose which was specifically labelled with ^{14}C in the carbon 1- or 6-position (Hers 1955, Landau & Merlevede 1963, Rauschenbach & Lamprecht 1964). The interpretation of these investigations was based on the fact that glycerol is biologically asymmetric, albeit chemically a symmetrical molecule (Swick & Nakao 1953, Schambye et al. 1954, Bublitz & Kennedy 1955). A consequence of this is that ^{14}C in the 3-position of D-glyceraldehyde (which originates from the 6-position of fructose 1-phosphate) is recovered in the 1-position of dihydroxyacetone phosphate when D-glyceraldehyde is reduced to glycerol, which in turn is phosphorylated to L-glycerol 3-phosphate by the glycerokinase. This molecule is then oxidized by the glycerophosphate dehydrogenase to dihydroxyacetone phosphate:





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Fig. 1. Metabolic pathways of fructose in the liver.

The dotted line indicates that phosphorylation of fructose by some "fructose 6-kinase" takes place in perfused livers of fasted pigs.

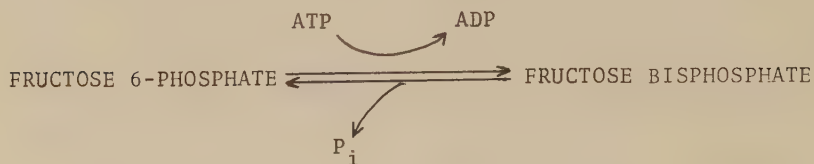
Thus recovery of ^{14}C from fructose labelled in the 6-position in the carbon atoms 3 and 4 in glucose revealed a measure of the importance of the glycerol pathway in the metabolism of D-glyceraldehyde. In this respect the observations were however inconsistent (cf. Landau & Merlevede 1963 and Rauchenbach and Lamprecht 1964).

The finding of a recovery of ^{14}C in both glucose carbon 1 and 6 following application of fructose specifically labelled in carbon 1 or 6 was interpreted as a consequence of isomerisation at the triose phosphate level (Hers 1955):

DIHYDROXYACETONE PHOSPHATE $\longleftrightarrow$ D-GLYCERALDEHYDE 3-PHOSPHATE

and it was also thought to prove that the activity of enzymes which phosphorylate fructose directly in the 6-position is negligible. It has however recently been shown that this is not always the case (Lundquist et al. 1974). In the isolated pig liver which is perfused at 38°C after a period of pre-cooling, only about 10% of the fructose taken up is phosphorylated in the carbon 1-position, whereas 90% enters the common glycolytic pathway presumably via phosphorylation in the carbon 6-position. The distribution of ^{14}C from specifically labelled fructose in carbon 1 and 6 in glucose is however similar to that found in cases in which fructose 1-phosphate must be assumed to be an obligatory intermediary metabolite. We are thus forced to accept isomerisation at the triose phosphate level even in cases in which fructose is phosphorylated in the 6-position. This

is feasible only if there is a considerable activity of substrate cycling of fructose 6-phosphate:



and if fructose biphosphate is readily cleaved by the aldolase (cf. Fig. 1). This has been shown to occur in hind limbs of pigs suffering from malignant hyperthermia (Clark et al. 1973), and to be involved in the maintenance of body temperature in the bumble bee (Clark et al. 1973). From the studies with pig liver thus two problems emerge : 1) By which enzyme may fructose be phosphorylated in the 6-position, and 2) to what extent is the above mentioned futile cycle active in normal liver. The last question may imply a reevaluation of the interpretation of fructose metabolism based on studies with specifically labelled fructose, as some substrate cycling of fructose-6-phosphate actually has been found also in rat hepatocytes (Rognstad et al. 1973).

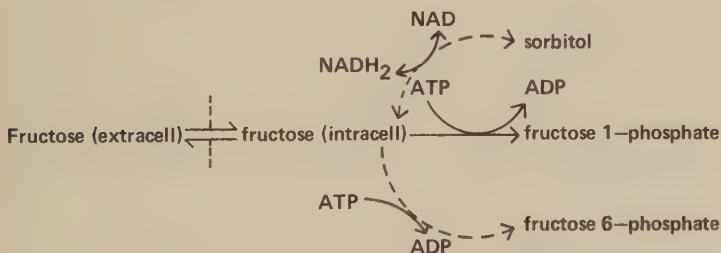
The metabolic scheme on which the interpretations in this paper is based is shown in Fig. 1. The suggestions of Muntz & Vanko, 1962, and Muntz 1968, that glucose and glycogen in part is synthesized from fructose via a pathway in which glucose 6-phosphate is not an obligatory precursor seems to be due to pitfalls in the interpretation of specific activities in a system which is not in steady state (Threlfall & Heath 1968) and is therefore not taken into

account (see also Antony et al. 1969 and Williams & Landau 1972).

REGULATION OF THE RATE OF FRUCTOSE UPTAKE.

The rate of uptake of a substance by the liver is determined by an interrelationship between the distribution between the vascular space and the interstitial space, the transmembrane transport and the intracellular metabolism. The extracellular distribution is dependent on blood flow. Small molecules have, however, been shown to be freely distributed in the extracellular water phase (Goresky 1970).

Fructose has previously been suggested to be distributed between the extra and the intracellular water phases by diffusion (Cahill et al. 1958), but this has been disputed recently (Sestoft & Fleron 1974 b). On the basis of the present knowledge the following sequence of reactions is expected to determine the rate of fructose uptake by the liver:



It has been shown that at a physiological NAD redox level in the cytosol, the reduction of fructose to sorbitol is of no sig-

nificance (Sestoft et al. 1972, Damgaard et al. 1973, Sestoft 1974b). In pig liver the magnitude of phosphorylation of fructose in the 6-position is an open question, whereas in rat liver the absence of inhibition of fructose phosphorylation by high glucose concentrations in the cell seems to indicate only a minor contribution of hexokinases to fructose phosphorylation (Sestoft 1974b). This is in accordance with the findings of Spolter et al. 1965 who found that inhibition of the aldolase resulted in a complete suppression of the formation of glucose 6-phosphate in rat liver homogenates. Furthermore, the kinetics of both hexokinase and glucokinase are unfavourable for phosphorylation of fructose (Walker 1965) and virtually no fructose phosphorylation takes place in cases in which the ketohexokinase activity is absent, e.g. in hereditary fructose intolerance (Froesch et al. 1959) and in the neonatal period in rat (Walker 1965, Ballard 1965), in man (Schwartz et al. 1964), and in pig (Swiatek 1971). Direct measurement of the flux of ^{14}C fructose to fructose 1-phosphate in rat liver did indicate that this pathway account for the metabolism of all fructose taken up (Damgaard & Sestoft, unpublished observation).

If pathways alternative to phosphorylation of fructose in the 1-position are neglected the rate of fructose uptake is determined by the kinetics of the membrane transport and the intracellular ketohexokinase reaction. During steady state the velocity expressions of these reactions may be equalized:

consider the kinetic constants of the membrane transport and of the intracellular phosphorylation separately.

The significance of the ketohexokinase reaction for the regulation of fructose metabolism.

The ketohexokinase is an enzyme which phosphorylates fructose only at the carbon 1-position (Slein et al. 1950, Adelman et al. 1967) and which has no activity with glucose (Slein et al. 1950, Leuthardt & Testa 1950, Cori et al. 1951). The K_m value of this enzyme with fructose and ATP, has been repeatedly determined on partially purified enzyme preparations and is about 0.5 mM with fructose (Hers 1952, Parks et al. 1957, Adelman et al. 1967, Sanchez et al. 1971) and 0.2 mM (Parks et al. 1957) or 1.5 mM with ATP (Adelman et al. 1967, Sanchez et al. 1971). The maximum activity measured in crude liver extracts is between 2.2 (Heinz et al. 1968) and 3.1 $\mu\text{mol}/\text{min}$ per g liver wet wt. (Adelman et al. 1967, Woods et al. 1970). The kinetic constants found with extracts from liver of the same strain of animals as used for perfusions are shown in Table II. The kinetics of rat liver ketohexokinase disagree with findings by others only as regards the K_m of ketohexokinase with ATP.

As several other kinases, the ketohexokinase is inhibited by ADP (Parks et al. 1957) with a K_i value of 1.7 mM (Sanchez et al. 1971). The product, fructose 1-phosphate (10 mM) has been shown to inhibit the ketohexokinase reaction completely (Froesch et al. 1959), but this was not found with different

Table II Kinetic constants of the ketohexokinase reaction.

The measurements were performed with liver high speed supernatant at 22° C as described, a) Sestoft & Fleron, 1974, b) Thieden et al. 1972, c) Sestoft et al. 1972. Values are means $\pm$ S.E.M. with the number of observations in parentheses.

	K_m with fructose (K_2)	K_m with ATP (K'_2)	V_{max}
	mM	mM	$\mu\text{mol/min} \times \text{g}$
Rat liver	0.46 ± 0.06 (3) a	0.30 ± 0.03 (4) a	3.0 ± 0.1 (6) b
Pig liver	0.5 (2) c		1.9 ± 0.4 (4) c
Human liver			0.9 (2) b

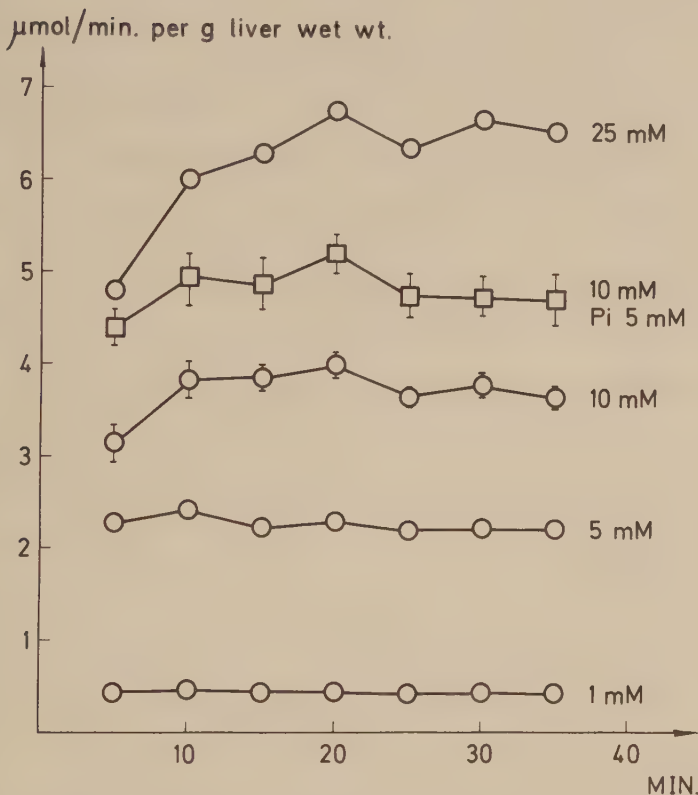
assay methods (Slein et al. 1950, Sestoft 1974b). Under physiological conditions the rate of fructose uptake by the perfused rat liver is independent of the concentration of fructose 1-phosphate (Sestoft 1974b).

The inhibition by ADP is partially reversed by K^+ , which apparently acts as an allosteric activator (Sanchez et al. 1971). An increase of K^+ concentration in the cuvette from 27 to 108 mM increased the K_m of the ketohexokinase reaction with fructose from 0.46 (Table II) to 0.53 and 0.8 mM found in two experiments (Sestoft & Fleron 1974b, see also Sanchez et al. 1971). Ethanol (10 mM), sorbitol (10 mM) and L-glycerol 3-phosphate (6 mM) did not inhibit the ketohexokinase reaction (Sestoft 1974b).

Under near physiological conditions the activity of ketohexokinase seems to be determined exclusively by the concentration of the two substrates (S_I & S_{ATP} equation (1)) during steady state. In the initial period after addition of fructose to a medium an inhibition is however seen, the duration and magnitude of which is dependent on the concentration of fructose (Fig. 2) (Sestoft 1974a). This may be attributable to a concomitant release of K^+ (Folke, M. unpublished observation) which may increase the inhibition by ADP. It seems not to be directly related to changes in the ATP or ADP concentrations which are similar under initial and steady state conditions (Sestoft 1974b).

Fructose induces a severe depletion of ATP in the liver (Raivio et al. 1969). The decrease in the ATP concentration during steady state fructose metabolism in the liver is approximately proportional to the rate of fructose phosphorylation (Table II). As the concentration of ATP decreases to values below the K_m of the ketohexokinase with ATP, the rate of fructose

Fructose elimination as a function of
time after fructose administration.



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Fig. 2. Fructose elimination as a function of time after administration of fructose.

This figure illustrates the relation between fructose concentration and the rate of fructose uptake, the lower rate of fructose uptake in the initial period at high fructose concentrations and the effect of a high concentration of inorganic phosphate on the rate of fructose uptake.

phosphorylation must be regulated by the concentration of ATP. As a consequence of this an increase in the ATP concentration from about 1 to about 1.5 $\mu\text{mol/g}$ caused a 25% increase in the rate of fructose uptake at unchanged intracellular fructose concentrations (Sestoft 1974 b). The kinetics of ketohexokinase with ATP also explain the decrease in the rate of fructose uptake induced by ethanol (Papenberg et al. 1970, Sestoft 1974 b) and by phenformin both of which cause an extra decrease in the hepatic ATP concentration (Lindros & Hillbom 1971, Haeckel 1973).

Thus the fructose induced depletion of ATP causes a decrease of the maximum rate of fructose uptake which can be attained. However, in the once-through perfused rat liver, the maximum rate of uptake (6.6 $\mu\text{mol/min per g}$) was more than twice the maximum activity measured in vitro (cf. Table II). This seems not to be due to phosphorylation of fructose by means of other kinases since there is no inhibition of glucose phosphorylation in cases in which the glucose concentration is 5 times that of fructose in the liver tissue (Sestoft 1974 b). Correction of the maximum rate of fructose uptake to a saturating ATP concentration assuming a K_m of 0.8 mM reveals a maximum activity ($V_{\max}$) of the fructose phosphorylating enzyme of 10.1 $\mu\text{mol/min per g}$ liver in the perfused organ (Sestoft & Fleron 1974 b). The fructose induced effect on the ATP concentration thus exerts a negative feed-back on the rate of fructose phosphorylation which is reduced by up to 30%.

The rate of fructose uptake in pig liver calculated per g wet weight, is not influenced by fasting (Sestoft et al.

1972, Lundquist et al. 1974) whereas in rat liver the uptake is increased by 25% (Sestoft 1974 b)(Fig. VII). The same has been found in rat liver slices (Stewart & Thompson 1941), but the phenomenon is not in agreement with observations made with crude extracts from rat liver in which the activity of fructose phosphorylation is 25% decreased by fasting 48 hours (Adelman et al. 1966). This seems not to be due to involvement of glucokinase the activity of which decreases severely by fasting (Sharma et al. 1964) since the fructose concentrations employed in vitro were so low that no fructose 6-phosphate was formed (Adelman et al. 1966). It is an open question whether the discrepancy is due to pitfalls in the measurements of enzyme activities.

As regards pig liver, fasting has been shown not to influence the activity of ketohexokinase measured in vitro (Swiatek 1971). The very small activity of this enzyme in the perfused liver (Lundquist et al. 1974) therefore must be explained in some other way, for instance by lack of ATP.

Fructose feeding in itself does not induce significant changes in the activity of the ketohexokinase (Adelman et al. 1966, Heinz et al. 1968, Hirasawa et al. 1972).

Concluding it can be said that the ketohexokinase is remarkably unregulated.

The significance of the membrane transport for the regulation of fructose uptake.

Although the K_m of ketohexokinase with fructose is below 1 mM, saturating concentrations of fructose is not attained in perfusion experiments with less than 25 mM fructose in the medium (Sestoft et al. 1972, Assimacopoulos-Jeannet et al. 1973, Sestoft 1974 b). This fact has not been taken into account in several recent perfusion studies in which the rate of fructose uptake accordingly has been decreasing during the perfusion period (Hems et al. 1966, Papenberg et al. 1970, Woods et al. 1970).

The finding of a steep gradient of fructose concentration across the cell membrane (Woods et al. 1970, Sestoft & Fleron 1974 a) (Table III) indicated that the regulation of the intracellular fructose concentration (S_I in equation (1a)) should be of importance for the regulation of the net uptake of fructose by the perfused organ. The very large difference between extra- and intracellular fructose concentrations is maintained only in the low concentration interval whereas at high fructose concentration a reduced and constant concentration gradient is observed (Table III). These findings are in accordance with a carrier mediated transport of fructose across the cell membrane combined with intracellular phosphorylation with the kinetic properties as described above (Table II). The K_m of the membrane transport (K_1 equation (1b)) is 67 mM and V_{max} (V_1) 30 $\mu\text{mol/min}$ per g liver (Sestoft & Fleron 1974 b).

Table III. General table for fructose elimination by perfused rat liver.

Livers of fed rats weighing 170 g were perfused at a flow of 5 ml/min, i.e. 0.72 ml/min per g liver. Fructose concentration in the affluent and in the effluent medium are given as the concentration in the waterphase. The first four columns are the observed values on which the calculation of the elimination kinetics is based. The calculated extra- and intracellular fructose concentrations are given as the concentrations in the respective water phases. Calculations performed according to Sestoft & Fleron 1974 b.

Fructose in the affluent (mM)	Fructose in the effluent (mM)	Fructose in biopsies ($\mu\text{mol/g}$)	ATP in biopsies ($\mu\text{mol/g}$)	Extracellular fructose (mM) mean	Intracellular fructose (mM) mean	Rate of uptake ($\mu\text{mol/min per g}$)	Influx $\mu\text{mol/min per g}$	Efflux $\mu\text{mol/min per g}$
1.49	0.65	0.14	2.00	1.02	0.05	0.43	0.45	0.02
1.49	0.65	0.05	2.33	1.03	0.05	0.44	0.46	0.02
7.37	2.92	0.69	1.86	5.10	0.27	2.03	2.15	0.12
7.44	3.39	0.68	1.33	5.21	0.28	2.06	2.19	0.13
14.57	6.81	2.01	1.29	10.55	0.76	3.80	4.13	0.34
14.71	7.96	2.60	1.05	10.66	0.81	3.81	4.17	0.36
15.10	7.93	2.31	1.11	10.89	0.83	3.88	4.25	0.37
15.40	7.62	2.38	1.17	10.93	0.82	3.89	4.26	0.37
35.89	22.33	10.61	0.83	29.62	7.31	6.33	9.32	2.99
36.81	22.79	10.70	0.75	30.73	8.35	6.19	9.56	3.37
114.20	104.44	61.84	0.34	109.92	58.79	4.70	18.92	14.22
129.36	114.20	61.17	0.56	123.80	55.70	5.95	19.76	13.81

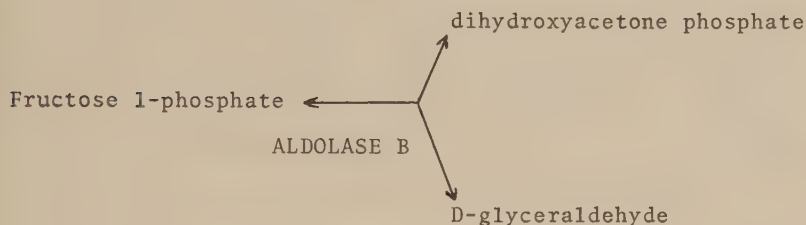
As K_2 is about 100 times lower than K_1 changes in the concentration of S_I in the low concentration interval will obviously influence the rate of phosphorylation (1a) much more than the efflux of fructose (1b, last term). Therefore the net uptake of fructose is largely determined by the rate of membrane transport at fructose concentrations below 25 mM in the affluent medium. At high concentrations the $V_{\max}$ for the intracellular phosphorylation solely determines the rate of fructose uptake. The above is of course only rough estimates of what determines the rate of fructose uptake, since no term in equation (1) can be considered separately. However, in one respect the membrane transport process seems to be of overwhelming importance: If the membrane transport were not rate limiting for the fructose uptake at low fructose concentrations, ingestion of even small amounts of fructose would cause a severe depletion of adenine nucleotides from the liver. Phosphorylation of fructose by hexokinase (or by glucokinase) should be expected under conditions with a high intracellular fructose and a low glucose concentration. Due to the steep concentration gradient for fructose across the cell membrane and the ready glucose formation from fructose such a condition will only occur transiently.

REGULATION AT THE FRUCTOSE 1-PHOSPHATE ALDOLASE LEVEL.

In contrast to the phosphorylation of fructose by ketohexokinase, the aldolase catalyzed cleavage of fructose 1-phosphate is subjected to regulatory inhibition by a large

number of metabolites (cf. Woods et al. 1970).

The cleavage of fructose 1-phosphate by the liver aldolase (B) is a reversible process which greatly favours the aldol condensation (Meyerhof et al. 1936, Lehninger et al. 1955)



Some kind of inhibition of the aldolase cleavage seems to be the cause of one of the most characteristic phenomena of fructose metabolism, the accumulation of fructose 1-phosphate, which has been found to occur in several species (Kjerulf-Jensen 1942, Burch et al. 1970, Sestoft et al. 1972).

In pig liver experiments it was clearly shown that the inhibition of the aldolase is connected to phosphorylation of fructose, since a burst of D-glyceraldehyde was found in the very initial period after addition of fructose to the medium and in the period after the fructose elimination had ceased (Sestoft et al. 1972). This rules out D-glyceraldehyde as an important inhibitor under physiological conditions at least in this species (cf. Spolter et al. 1965, Woods et al. 1970). The mechanism of aldolase inhibition was directly related to the phosphorylation of fructose by Woods et al. 1970. This is

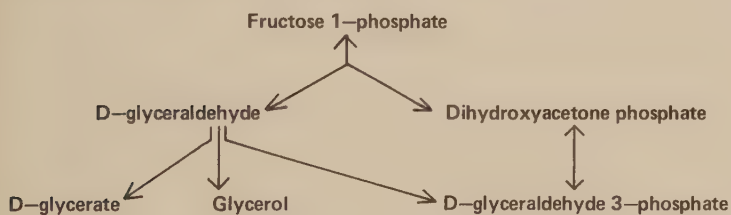
corroborated by the findings that a burst of carbon into the pools of glycolytic intermediary metabolites is seen in cases in which fructose 1-phosphate is metabolized without simultaneous phosphorylation of fructose (Sestoft 1974 a). The degradation of AMP was shown to be increased during fructose metabolism in rat liver by Raivio et al. 1969. AMP is deaminated to IMP and this intermediary metabolite, which is strongly inhibitory to the fructose 1-phosphate cleavage by liver aldolase, was shown to increase to inhibitory concentrations in the initial period of fructose metabolism (Woods et al. 1970). However, a large series of metabolites are inhibitory to this reaction, and it has been shown that the accumulation of fructose 1-phosphate also takes place in cases in which no increase in the IMP concentration is expected in liver (high P_i in the medium) (Woods 1972, Sestoft 1974 b). That the inhibition of fructose 1-phosphate cleavage is not confined to the regulation by a single product is also supported by the finding of differences in the magnitude of inhibition in different nutritional states (Sestoft 1974 a). The smaller accumulation of fructose 1-phosphate during fructose metabolism in livers of fasted rats cannot be ascribed to dietary or hormonal changes in the activity of the fructose 1-phosphate aldolase (Adelman et al. 1966).

The reverse reaction, the aldol condensation to fructose 1-phosphate, seems not to occur to any considerable extent under physiological conditions since perfusion with D-glyceraldehyde in concentrations above 1 mM did not give rise to

any significant increase in the concentration of fructose 1-phosphate in pig liver (Sestoft et al. 1972).

INITIAL STEPS OF D-GLYCERALDEHYDE METABOLISM.

As fructose 1-phosphate, D-glyceraldehyde is a metabolite which is unique for the metabolism of fructose in tissues which phosphorylate fructose in the carbon 1-position, D-glyceraldehyde may undergo phosphorylation by triokinase (Hers & Kusaka 1953) reduction by alcohol dehydrogenase (NAD) (Wolf & Leuthardt 1953, Holzer et al. 1955) reduction by alcohol dehydrogenase (NADP) (Moore 1959) oxidation by aldehyde dehydrogenase (Holldorf et al. 1959), or aldol condensation with dihydroxyacetone phosphate (Meyerhof et al. 1936). The Michaelis constants of these reactions are given in Table IV.



Of these reactions only the aldol condensation is reversible under physiological conditions.

Liver tissue has a high activity for metabolism of D-glyceraldehyde (Rosenthal 1930, Sestoft et al. 1972). The

Table IV. Michaelis constants of enzymes involved in the metabolism of D-glyceraldehyde.

The figures for rat and human liver enzymes are from Thieden et al. 1972, and for pig liver enzymes from Sestoft et al. 1972.

	K_m (mM)			Substrate
	Rat liver	Pig liver	Human liver	
Alcohol dehydrogenase (NAD)	8–10	18	80–90	D-glyceraldehyde
Alcohol dehydrogenase (NADP)	0.6 ^x	4.7	2.5–3.3	—
Aldehyde dehydrogenase (NAD)	0.31–0.40	0.12	0.16	—
Triokinase (ATP)	0.014–0.017	—	0.1	—
Glycerate kinase (ATP)	0.03	— ^{xx}	2.5–3.0	D-glycerate

^x Moore, 1959.

^{xx} The K_m of glycerate kinase in pig liver could not be determined because the activity of the enzyme was very low.

maximum activity cannot be measured since at high concentrations of D-glyceraldehyde the uptake is inhibited by the substrate (Rosenthal 1931). The rate of D-glyceraldehyde elimination as a function of the substrate concentration reveals a complicated pattern, which has not been analyzed, but which may well be a sum function of kinetics of membrane transport with a high K_m value, and of kinetics of the enzymes involved in D-glyceraldehyde metabolism. In pig liver the processes for D-glyceraldehyde metabolism outlined above seem to take place except the aldol condensation (Sestoft et al. 1972).

The D-glyceraldehyde metabolism as a part of fructose metabolism has been investigated in experiments in which the fate of (6-¹⁴C) fructose was measured. The conflicting results seem to be explained in part by factors which influence the relative importance of the different pathways, but which have not been kept under control or even measured in these investigations (Hers 1955, Landau & Merlevede 1963, Rauschenbach & Lamprecht 1964, Hue et al. 1972). The factors are, the concentration of D-glyceraldehyde attained, the NAD and NADP redox level in the cytosol, the time elapsed after addition of fructose (see page 39), and the concentration of ATP. However, these investigations show that metabolism via as well glycerol and glycerate as D-glyceraldehyde 3-phosphate takes place. The concentration of D-glyceraldehyde under fructose metabolism is low in both pig and rat liver (Heinz & Jung-hänel 1969, Woods et al. 1970, Sestoft et al. 1972, Sestoft 1974 b), and since the rate of fructose metabolism at 25 mM

fructose is about 6 $\mu\text{mol/min}$ per g liver the flux of carbon through the small D-glyceraldehyde pool (0.1 $\mu\text{mol/g}$) must attain the same value.

METABOLISM OF D-GLYCERATE.

The existence of a D-glycerate kinase phosphorylating D-glycerate in the carbon 2-position has been demonstrated in rat liver (Ichihara & Greenberg 1957, Holzer & Holldorf 1957, Lamprecht et al. 1959, Kattermann et al. 1961), and a production of D-glycerate from D-glyceraldehyde takes place in rat liver homogenates (Fig. 3). However, the quantitative significance of the D-glycerate pathway for fructose metabolism has not been established. In pig liver the glycerate kinase is virtually absent (Ichihara & Greenberg 1957, Sestoft et al. 1972), and as a consequence of this, D-glycerate accumulates during recirculating perfusion with fructose (Sestoft et al. 1972). The demonstration that glycerate kinase is also absent in human liver (Heinz et al. 1968) could, however, not be confirmed by us (Thieden et al. 1972).

Alternatively D-glycerate may be oxidized by a dehydrogenase linked to NAD and NADP to hydroxypyruvate (Heinz et al. 1962, Willis & Sallach 1962). This pathway seems to have some significance in intact tissue (Dickens & Williamson 1960).

METABOLISM OF ADENINE NUCLEOTIDES DURING FRUCTOSE METABOLISM.

The concomitant very rapid phosphorylation of fructose and accumulation of the phosphorylated product, fructose 1-phosphate, causes a decrease in the tissue concentration of

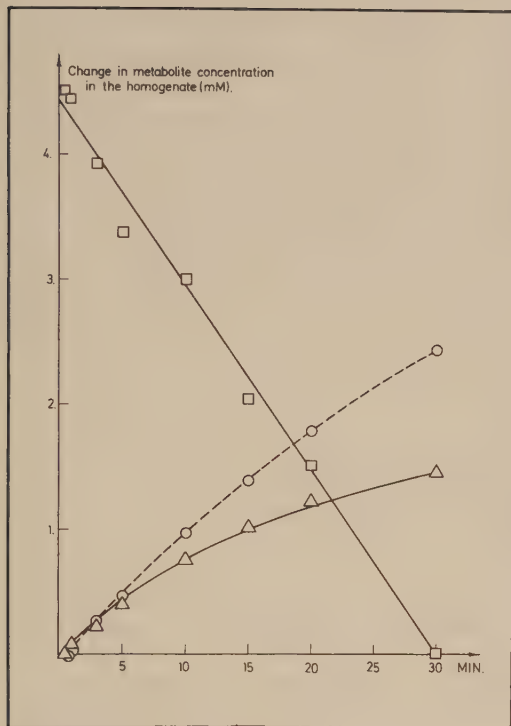
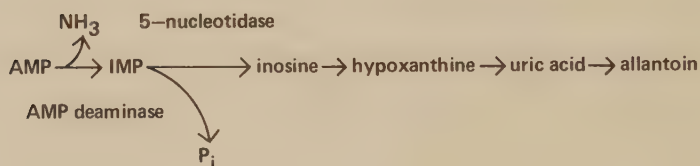


Fig. 3. Dismutation of D-glyceraldehyde to glycerol and D-glycerate in rat liver high speed supernatant.

□ D-glyceraldehyde, △ Glycerol, ○ D-glycerate. After homogenization the 10% rat liver homogenate was centrifuged for 10 min at 10 000 rpm. (121 000 g x min). The supernatant was dialyzed in order to deplete it for ATP. The supernatant was then incubated for 30 min at 37° C in a phosphate buffer with atmospheric air. Then 0.3 mM NAD⁺ was added and after 10 min D-glyceraldehyde was added to a concentration of 10 mM, and the changes in the concentrations of the three metabolites were followed for 30 min. The figure illustrates the ability of a rat liver high speed supernatant without mitochondria, and depleted for ATP to produce glycerol and D-glycerate from D-glyceraldehyde. The increase in the concentrations of D-glycerate and glycerol accounts for 90% of the decrease in the concentration of D-glyceraldehyde. (Damgaard & Sestoft, unpublished observation).

both ATP and inorganic phosphate (Raivio et al. 1969, Burch et al. 1970, Woods et al. 1970, Sestoft 1974 a). The concentration of total adenine nucleotides also decreases, and the rate of AMP degradation increases leading to an increased release of the final products of the metabolic sequence, uric acid or allantoin (Perheentupa & Raivio 1967, Mäenpää et al. 1968, Raivio et al. 1969).



This increase in AMP degradation has tentatively been thought to be caused by changes in the pattern of inhibition of AMP deaminase and of 5-nucleotidase (Woods et al. 1970). The AMP deaminase is inhibited by P_i (Nikiforuk & Colowick 1956) and the 5-nucleotidase by ATP (Baer et al. 1966) and during fructose metabolism the concentration of both inhibitors are severely decreased, leading to an increased AMP degradation (Woods et al. 1970). Several facts, however, seem to suggest a much more complicated pattern of regulation. The inhibition of AMP deaminase by P_i is reduced at physiological concentrations of KCl, whereas the inhibitory effect of several phosphorylated intermediates such as the glycerate phosphates, is enhanced (Sammons et al. 1970). Furthermore both the liver and muscle AMP deaminases are activated by ATP (Lee & Wang 1968, Sammons et al. 1970) and the presence of

ATP changes the inhibition by P_i from a competitive to an uncompetitive one and decreases its magnitude (Lee & Wang 1968).

When applied to the conditions in the intact liver it can be stated that inorganic phosphate seems to play a role in the metabolism of AMP since the concentration of IMP is not increased when a high concentration of P_i has been employed (Woods 1972). The differences in accumulation of fructose 1-phosphate between fed and fasted animals could be thought to be caused by differences in AMP deaminase inhibition of liver aldolase caused in part by differences in the concentration of the glycerate phosphates (Sammons et al. 1970, Sestoft 1974 a). This, however, implies that differences in inhibition of fructose 1-phosphate aldolase should be due to differences in the concentration of IMP, and this is in conflict with the fact that accumulation of fructose 1-phosphate takes place also when the concentration of IMP is not elevated as pointed out by Sestoft 1974 a & 1974 b.

Another consequence of the depletion of adenine nucleotides is a release of Mg^{++} (Levin et al. 1963, Mäenpää 1972), caused partly by the decrease in total mononucleotides, to which Mg^{++} is bound in a complex form (cf. Rose 1968), partly due to a relative increase in the concentration of AMP and ADP which compared to ATP are less strong complex binders.

CHANGES IN ENZYME REGULATIONS INDUCED BY FRUCTOSE.

The most conspicuous features of fructose metabolism are the accumulation of fructose 1-phosphate and the decrease in the concentration of ATP.

Fructose 1-phosphate in concentrations comparable to those found under physiological conditions exhibits regulatory properties with a number of enzymatic reactions in vitro (Table V). Among these regulations the activation of pyruvate kinase (Eggleston & Woods 1970), the inhibition of glycogen phosphorylase a (van den Berghe et al. 1973) and the absence of any significant effect on fructose biphosphate aldolase (Spolter et al. 1965) are the most important ones for the interpretation of metabolic consequences of fructose administration to intact liver.

The decrease in the ATP concentration in itself may influence the activities of kinases with a high K_m for ATP, e.g. triokinase (Frandsen & Grunnet 1971) and glycerate kinase (Thieden et al. 1972). The decrease in ATP concentration may also be expected to influence enzymatic reactions of generalized importance for the cell metabolism, e.g. maintainance of the ionic gradient across the cell membrane by influencing the ATPase connected with the $\text{Na}^+ - \text{K}^+$ pump (van Rossum 1972) and the synthesis of cyclic AMP from ATP via the adenyl cyclase (van den Berghe et al. 1973).

Generally the decrease in the cellular concentration of ATP causes a decrease in synthetic processes as albumin

Table V. Regulatory effects of fructose 1-phosphate on liver enzymes in vitro. In the case of AMP deaminase the reference is to the chicken muscle enzyme.

Enzyme	Inhibition by fructose 1-phosphate	Species	Reference
Ketohexokinase	Total inhibition at 10 mM	Rat	Froesch et al. 1959
Aldolase (fructose biphosphate)	Inhibition by 22 mM	Rabbit	Spolter et al. 1965
Aldolase (fructose biphosphate)	Inhibition (50%) at 10 mM	Rat	Froesch et al. 1959
Hexose phosphate isomerase	Inhibition (50%) at 1.5 mM	Rabbit	Zalitis & Olivier 1967
Phosphorylase a	Inhibition (50%) at 3 mM	Mouse	van den Berghe et al. 1973
Phosphoglucomutase	Inhibition (50%) at 0.8 mM	?	Sidbury 1959
Pyruvate kinase	Activation by fructose 1-phosphate Activation (400%) by 10 mM	Rat	Eggleston & Woods 1970
Ketohexokinase	No effect of fructose 1-phosphate ?	Rat	Slein et al. 1950
Ketohexokinase	10 mM	Rat	Sestoft 1974 b
Fructose biphosphatase	No protection against ATP inactivation	Rabbit	Taketa et al. 1971
AMP deaminase	Slight inhibition (5%) at 3 mM	Chicken	Sammons et al. 1970
Phosphorylase kinase		Mouse	van den Berghe et al. 1973
Amylo 1,6-glucosidase		Mouse	van den Berghe et al. 1973

or RNA synthesis (Mäenpää et al. 1968, Bode et al. 1973).

The ATP to ADP concentration ratio has repeatedly been shown to decrease during fructose metabolism (Raivio et al. 1969, Woods et al. 1970, Sestoft 1974 a), and this may cause an inhibition of some kinases, e.g. glycerol kinase (Bublitz & Kennedy 1954, Grunnet & Lundquist 1967). The concentration of ADP following fructose administration has been found either to increase transiently in vivo (Raivio et al. 1969), to decrease in a recirculating perfusion system (Woods et al. 1970) or to remain unchanged in a non-recirculating perfusion system in which a constant fructose concentration is maintained (Sestoft 1974 a). The regulatory properties of this mononucleotide will therefore only be considered in connection with the remarks on energy metabolism. (See also page 13).

The concentration of AMP has been found to increase transiently in the initial 5 min period after addition of fructose in vivo and in a non-recirculating perfusion system (Raivio et al. 1969, Sestoft 1974 a). Whether this is of any significance for the regulation of carbon flux from fructose to glucose (cf. page 39) as well as the regulatory role of AMP for the phosphofructokinase and fructose biphosphatase steps in vivo is ambiguous (cf. Taketa et al. 1971). Recent evidence of substrate cycling at this level (Clark et al. 1973, Rognstad et al. 1973) seems to indicate that the direction of the net process is under some conditions a resultant of two rapid processes in opposite directions. Therefore a small inhibition or activation of one of the processes may result in a large increase in the net process although the inhibition

or activation is impossible to detect with the purified enzyme (cf. Start & Newsholme 1970).

The inhibitory action of IMP on fructose 1-phosphate aldolase has been discussed previously (page 24).

REGULATION OF THE RATE BY WHICH PRODUCTS ARE RELEASED DURING FRUCTOSE METABOLISM.

It is well documented that glucose as well as lactate is released at a high rate from fructose metabolizing livers of both fed and fasted animals. Further the metabolism of fructose is characterized by production of metabolites which are unique for fructose metabolism, i.e. sorbitol, D-glyceraldehyde and D-glycerate. The rate of release of different products depends on a) the composition of the perfusion medium, b) the time after initiation of fructose metabolism in the liver, c) the cytosolic NAD redox level of the liver and d) various particular circumstances as hormone treatment or addition of drugs etc. which will not be considered here.

a. Composition of the perfusion medium.

The rate of product release depends on the rate of fructose uptake during steady state (Fig. 4). There is a proportionality between fructose uptake and glucose and lactate release. In these livers no glycogen formation takes place (Sestoft 1974 b) because glycogen formation in perfused liver requires about 10 mM glucose in the affluent medium (Ross et al. 1967). In cases with no or moderate amounts of glucose added to the affluent medium the glucose release accounts for

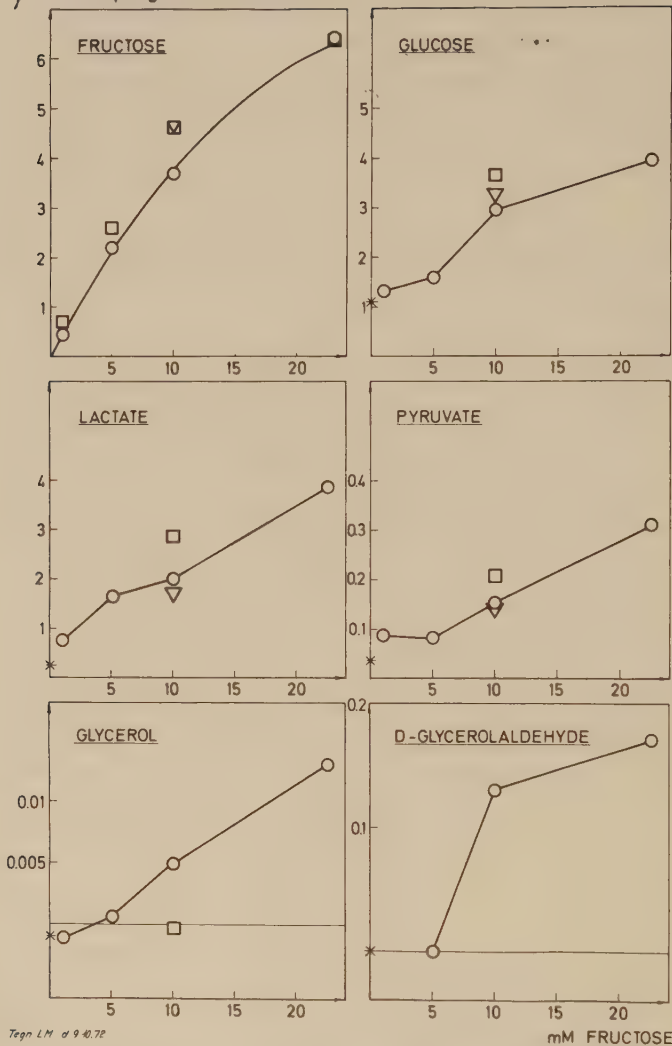
μ mol/min. per g liver wet wt.

Fig. 4. The rate of fructose uptake and the rate of release of main products by perfused rat liver as a function of the fructose concentration in the affluent medium under steady state conditions.

The star on the ordinate indicates the rate of release under conditions with no fructose added. $\circ$ fed animal P_i 1 mM, $\square$ fed animal P_i 5 mM, ∇ fasted animal P_i 1 mM.

Table VI. The influence of the glucose concentration in the medium on the rate of glucose and lactate release at 10 mM fructose in the affluent medium. Fed rats. Values are given $\pm$ S.E.M. (n = 3). (Sestoft, unpublished results).

Affluent medium	glucose release	lactate release
	$\mu\text{mol}/\text{min per g liver}$	
0 mM glucose, 0 mM fructose added	0.96 ± 0.16	0.61 ± 0.28
0 mM glucose, 10 mM fructose added	2.66 ± 0.19	1.97 ± 0.15
5 mM glucose, 10 mM fructose added	1.62 ± 0.14	2.50 ± 0.19
10 mM glucose, 10 mM fructose added	0.74 ± 0.43	2.81 ± 0.33
25 mM glucose, 10 mM fructose added	-0.41 ± 0.36	2.82 ± 0.21

about 3/4 of the fructose carbon taken up and the lactate release for about 1/4 (Woods et al. 1970, Sestoft 1974 b). The same figures have been found in vivo (Froesch et al. 1971). With an increasing concentration of glucose in the affluent medium a decrease in the percentage of fructose uptake accounted for by glucose release is seen (Table VI), and the total amount of fructose carbon accounted for by the release of glucose plus lactate decreases to about 50%. The oxidation of fructose to CO_2 only accounts for a few percent (Kruhøffer & Muntz 1954) and so does the fatty acid synthesis (Topping & Mayes 1972, Damgaard et al. 1974), and therefore a net glycogen synthesis must have taken place. These considerations are important for the interpretation of previous investigations which have been performed using either recirculating perfusion systems (Ross et al. 1967, Exton & Park 1969, Woods et al. 1970, Topping & Mayes 1972, Sestoft et al. 1972) in which the glucose concentration in the medium increases during the perfusion period (cf. Sestoft et al. 1972) or in vivo experiments in which the effect of fructose on the metabolite release is influenced by hormones released during anesthesia (stress) which tend to increase the concentration of glycolytic intermediates in the fed animal (Faupel et al. 1972). In one case it has been claimed that fructose causes no glucose production, no glycogen formation and inhibits the uptake of lactate by the liver although the rate of fatty acid synthesis is also low (Topping & Mayes 1972). A corollary of these considerations should be that all of the oxygen uptake should be

used for fructose oxidation, and the rate of oxygen uptake should be at least two times the value found by others in perfused livers.

In livers of fed animals fructose causes an overall increase in the rate of metabolite release including glycerol, D-glyceraldehyde, glycerate, pyruvate, citrate, malate, and ketone bodies (Heinz & Junghänel 1969, Sestoft et al. 1972, Sestoft 1974 b) and the concentration of most intermediary metabolites increases (Heinz & Junghänel 1969, Burch et al. 1970, Woods et al. 1970, Sestoft 1974 a).

In the fasted animals the same picture is seen except that fructose causes a significant reduction in the rate of ketone body release (Edson 1936, Wieland & Matschinsky 1962, Sestoft 1974 b). In strongly fasted animals in which there is a shortage of substrate for ketone body formation fructose causes an increase in the rate of ketone body formation (Söling & Willms 1970, Söling & Willms 1971) as it does in livers of fed animals (Sestoft 1974 b).

b. Time dependence of the rate and pattern of metabolite release after fructose. Pathogenesis of hypoglycemia.

The effect of fructose on glucose production by the liver can be studied in the perfused organ in which the rate of glucose release has been found to be markedly inhibited in the first 10 min period in the fed animal (Sestoft 1974 a & b). In the fasted animal the rate of glucose release increases immediately after addition of fructose, and during steady

state the release of glucose is of the same magnitude in fed and fasted animals (Fig. 5). Measurement of gluconeogenesis from fructose over both initial and steady state periods may therefore lead to equivocal results (cf. Ross et al. 1967). The difference in carbon release between livers of fed and fasted animals is not due to differences in the rate of fructose uptake in the initial period (Fig. 5).

The bulk of fructose carbon is introduced into the common glycolytic pathway at the triose phosphate level. Therefore fructose (and dihydroxyacetone) has been used in order to investigate the regulation of gluconeogenesis by the enzymatic steps between triose phosphates and glucose (cf. Fig. 1). Except for a moderate effect of glucagon (Veneziale 1971) this part of the gluconeogenetic pathway has been found not to include enzymatic steps of importance for the overall rate of gluconeogenesis (Exton & Park 1969, Berry & Kun 1972). This has been confirmed with perfused livers of fasted animals (Sestoft 1974 a & b).

An explanation of the inhibition of glucose release from livers of fed animals must include an inhibition of both glycolysis and gluconeogenesis above the triose phosphate level. The demonstration by van den Berghe et al. 1973 of an inhibition of glycogen phosphorylase by fructose 1-phosphate provides a very likely explanation of the inhibition of glycogenolysis. Analysis of the pattern of intermediary metabolites above the triose phosphate level reveals an inhibition of fructose bisphosphatase (Sestoft 1974 a & b), but revealed no evidence

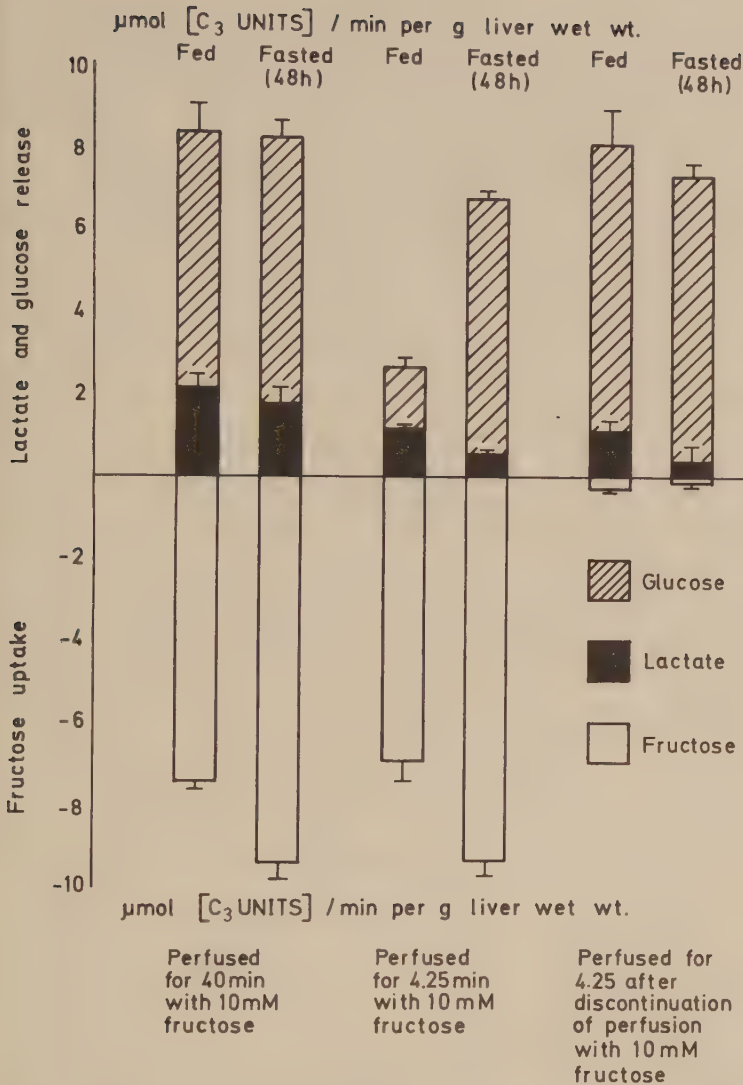


Fig. 5. The rate of fructose uptake and the rate of release of glucose and lactate by perfused livers of fed and fasted animals under different conditions.

The figure shows the low rate of glucose release by livers of fed animals in the initial period after fructose was given. Means $\pm$ S.E.M. (n=3).

of inhibition of the hexose phosphate isomerase which could have been anticipated by regarding the in vitro inhibition of this enzyme by fructose 1-phosphate (Zalitis et al. 1967). The inhibition of the gluconeogenic pathway at the fructose biphosphate level does not explain the gap between carbon uptake and carbon release in the livers of fed animals in the initial period after fructose (cf. Fig. 5). This is however fully explained by an increased accumulation of fructose 1-phosphate in livers of fed rats (Sestoft 1974 a).

The mechanism of the fructose biphosphatase inhibition remains obscure, but some suggestions may be made:

A) D-glycerate 3-phosphate reverses the inactivation of fructose biphosphatase by ATP and ADP (Taketa et al. 1971) and the concentration of glycerate 3-phosphate is three times higher in livers of fasted than of fed rats at the time of inhibition of gluconeogenesis (Sestoft 1974 a), B) the catalytic properties of fructose biphosphatase are influenced by lysosomal enzymes which may be liberated as a consequence of a decrease in the concentration of ATP (Pontremoli et al. 1973). This leads to a desinhibition of the enzyme which could explain the effect in fed rats, provided that the effect is delayed about 10 min.

In hereditary fructose intolerance, an inborn error of the development of fructose 1-phosphate aldolase activity in the liver fructose causes a severe hypoglycemia. A less pronounced effect has however also been observed in normal animals (Davidson et al. 1936) and in man (Bernstein & Holm 1922, Leuthardt 1960, Schwartz et al. 1964). This effect

develops in the very initial period after fructose administration and is not an insulin effect (Corvilain & Tagnon 1961).

Regarding the hypoglycemic effect and the strong initial inhibition of fructose 1-phosphate aldolase the conditions in perfused livers of fed rats are very similar to those in the liver of humans suffering from hereditary fructose intolerance. One hypothesis for the pathogenesis of hypoglycemia in this disease may then be: an inhibition of glycogenolysis by fructose 1-phosphate and an initial inhibition of fructose biphosphatase in fed subjects. In the fed subject the desinhibition of fructose biphosphatase by lysosomal enzymes may increase the rate of substrate cycling at the fructose 6-phosphate level (cf. page 10) thus giving rise to an extra depletion of the liver for ATP which is necessary for gluconeogenesis from the normal gluconeogenic precursors alanine, lactate etc.

The initial decrease in glucose output from livers of fed rats should be distinguished from the effect of fructose on the rate of glucose release measured over longer periods in the rat (Topping & Mayes 1972) and in man (Bergström and Hultman 1967). This effect seems to be related to an increased rate of glycogen formation which has been found in vivo (Cori 1926). The increased net rate of glycogen formation may be explained by the inhibition of glycogen phosphorylase by fructose 1-phosphate (van den Berghe et al. 1973) and a high concentration of glucose (cf. page 35).

c. The effect of the cytosolic NAD redox level on fructose metabolism.

Ethanol causes a prompt and persistent increase in the NADH_2/NAD ration in cytoplasm (Forsander et al. 1965, Veech et al. 1972) and rapid changes in a widely interlinked network of metabolites including lactate and pyruvate. The ratio between the concentrations of lactate and pyruvate is usually employed as a measure of the cytosolic NAD redox level.*)



At equilibrium the NADH_2/NAD ratio is related to lactate-pyruvate and other redox pairs as sorbitol-fructose in the following manner:

$$\frac{[\text{NADH}_2]}{[\text{NAD}]} = K_{\text{LDH}} \frac{[\text{lactate}]}{[\text{pyruvate}]} = K_{\text{SDH}} \frac{[\text{sorbitol}]}{[\text{fructose}]}$$

where K_{LDH} and K_{SDH} are the apparent equilibrium constants of the respective enzymatic reactions of lactate dehydrogenase and sorbitol dehydrogenase. From these equations it is evident that if both reactions are in equilibrium with the same cytosolic NAD pool, the following equation is true:

$$\frac{\text{lactate}}{\text{pyruvate}} \times \frac{\text{fructose}}{\text{sorbitol}} = \frac{K_{\text{SDH}}}{K_{\text{LDH}}}$$

*) for sake of clarity the hydrogenions are not taken into account.

where the last term is a constant. This has been shown to be the case in pig and rat liver (Damgaard et al. 1973, Sestoft 1974 b) and must be expected to be the case also in human liver (Tygstrup et al. 1965).

At a normal cytosolic NAD redox level no sorbitol is released from fructose metabolizing livers (Sestoft et al. 1972, Sestoft 1974 b) whereas at a high NAD redox level the kinetics of sorbitol release seems to conform to Michaelis-Menten kinetics with the intrahepatic fructose concentration as substrate reference (Sestoft 1974 b). The half maximum rate of release is attained at an intracellular fructose concentration which is 5 times lower than the K_m value of the sorbitol dehydrogenase measured in vitro (Blakley 1951), and the maximum rate of release is much lower than the maximum activity measured in vitro (Blakley 1951, Heinz et al. 1968, Thieden et al. 1972).

Ethanol given together with fructose causes a decrease in the rate of lactate release from fed rats (Sestoft 1974 b) and from fed and fasted pig livers (Damgaard et al. 1973, Lundquist et al. 1974)(Table VII). As the increase in the cytosolic NAD redox level would favour an increased rate of lactate release some inhibition of the carbon flux from fructose to lactate must take place. This has been confirmed in the perfused liver of fasted pigs (Lundquist et al. 1974). The cause of this decreasing lactate production is not an increased rate of pyruvate oxidation via Krebs cycle (Williamson et al. 1969) and not a decrease in the flux of carbon

Table VII. The effect of ethanol on glucose and lactate release during fructose metabolism. The rates were measured during steady periods of fructose metabolism. Negative values denotes uptake.

	RAT			PIG		
	$\mu\text{mol/min per g liver}$					
	Fed	Fasted	Fed + ethanol	Fed	Fasted	Fed + ethanol
Fructose uptake	-3.8	-4.7	-3.7	-2.4	-2.2	-2.7
Glucose release	3.1	3.2	3.5	1.0	1.0	0.53
Lactate release	2.2	1.8	1.6	1.0	0.6	0.33
						Fasted + ethanol
						1.4
						0.06

via the glycerate kinase as proposed by Tygstrup et al. 1965, since the same effect is seen in the pig, in which the glycerate pathway is nearly non-existing (Sestoft et al. 1972).

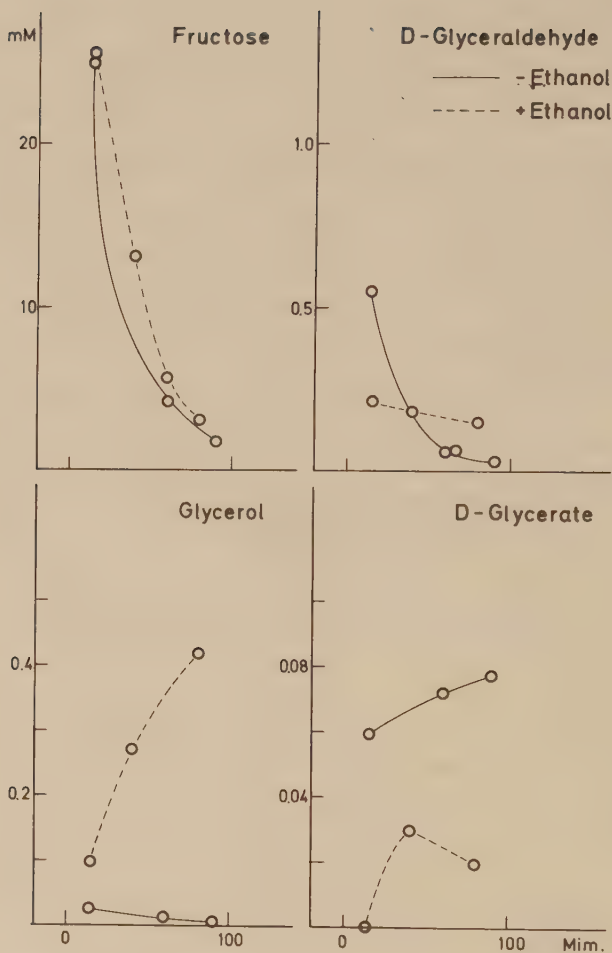
Accumulation of phosphorylated intermediary metabolites as e.g. L-glycerol 3-phosphate may explain the phenomenon in the period in which accumulation takes place (cf. Papenberg 1971). During steady state the direct effect of NADH on glyceraldehyde 3-phosphate dehydrogenase may provide an explanation (Furfine & Velick 1965) or the effect of the increased NADH_2/NAD on the equilibrium of this enzymatic reaction:

$$\frac{[\text{NADH}_2]}{[\text{NAD}]} = \frac{[\text{ADP}][\text{P}_i]}{[\text{ATP}]} \cdot \frac{[\text{glyceraldehyde 3-phosphate}]}{[\text{glycerate 3-phosphate}]} \cdot K$$

may play a role by changing the equilibrium in the direction of the reduced substrate.

Changes in the cytosolic NAD redox level also influence the fate of D-glyceraldehyde, the steady state concentration of which is increased by ethanol (Damgaard et al. 1973, Sestoft 1974 b). In pig liver a switch-over from oxidation to reduction of this substrate could be clearly demonstrated (Fig. 6). This confirms previous reports on the increased rate of glycerol production in homogenates (Hassinen 1964) or glycerol release in vivo, in perfused livers and in hepatocytes (Tygstrup et al. 1965, Berry 1971, Sestoft 1974 b) during fructose and glyceraldehyde metabolism.

Inhibition by ethanol of glucose release from livers of fasted rats metabolizing fructose (Krebs et al. 1969) could



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Fig. 6. The concentrations of fructose, D-glyceraldehyde, D-glycerate and glycerol in the perfusion medium during pig liver perfusion with fructose at different redox levels.

The lactate to pyruvate concentration ratio in perfusions was without ethanol 40 and with ethanol 120. The figure illustrates the change from predominant release of D-glycerate at a low cytosolic NAD redox level to glycerol at a high redox level.

not be confirmed in experiments with fasted pigs (Lundquist et al. 1974) or in fed rats (Sestoft 1974 b). The reason for this discrepancy may be that the decrease in carbon input from fructose caused by sorbitol production during ethanol metabolism was not taken into account by Krebs et al. 1969. However, it should be mentioned that ethanol has a clearly depressing effect on gluconeogenesis from other gluconeogenic substrates as lactate, alanine etc. (Madison 1968, Krebs et al. 1969, Kreisberg et al. 1971).

INFLUENCE OF FRUCTOSE ON ENERGY METABOLISM.

During steady state fructose causes a 25-30% increase in the rate of oxygen uptake by the liver in the cat (Lundsgaard et al. 1936) in man (Tygstrup et al. 1965) in the pig (Sestoft et al. 1972) and in the rat (Sestoft 1974 a & b). Assuming a P/O ratio of 3, this surplus in oxygen uptake is enough to provide the ATP necessary for the phosphorylation of 10 mM fructose. Although lactate production from fructose should provide ATP enough for fructose phosphorylation the concentration of ATP under anaerobic conditions is so low (Woods 1972) that the rate of phosphorylation is at least decreased to half the value found under aerobic conditions (Woods & Krebs 1971). A clear estimate of the rate of fructose phosphorylation during anaerobiosis can however not be given since the rate of sorbitol production was not measured by Woods and Krebs 1971. Uncoupling by 2,4-dinitrophenol causes a complete inhibition of fructose phosphorylation in pig liver (Damgaard et al. 1973).

Thus a normal rate of fructose phosphorylation seems to depend on an undisturbed function of oxidative phosphorylation.

On the other hand metabolism of fructose causes profound interference with the function of oxidative phosphorylation. The effect of fructose on inorganic phosphate and adenine nucleotide metabolism in the intact animal was clearly demonstrated by Raivio et al. 1969. These effects were related to the accumulation of fructose 1-phosphate (Burch et al. 1970) which in turn was ascribed to the changes in inorganic phosphate and adenine nucleotide metabolism (Woods et al. 1970). However, a clear distinction between steady state and initial conditions was not made. By considering the initial period in which phosphorylated intermediates (fructose 1-phosphate and L-glycerol 3-phosphate) accumulate, it could be demonstrated that the amount of phosphate bound as organic esters counterpoises the amount of phosphate taken up by the liver, plus the decrease in free inorganic phosphate and in anhydride bound phosphate in the adenine nucleotides (Sestoft 1974 a) (Table VIII).

The changes in ATP, ADP, inorganic phosphate and fructose 1-phosphate seem to be related to the initial effect of fructose on the rate of oxygen uptake which was demonstrated by Hassinen & Ylikhari 1970, and confirmed by others using different perfusion systems (Zehner et al. 1973, Sestoft 1974 a & b). The effect may be subdivided into three phases: I. a 10% increase in the rate of oxygen uptake in the initial 2 min period after addition of fructose. II. a 30% decrease in the

Table VIII. Phosphate balance at 4 min after addition of fructose (10mM) to the perfusate. After 26 min perfusion, fructose (10mM) was added to the medium, and after further 4 min the biopsy was performed. Controls were perfused for 26 min without fructose. The uptake of phosphate (0–4 min) was calculated as the integral of the uptake curve.

$\mu\text{mol/g liver (n = 3)}$						
	FED			FASTED		
	control	fructose	ΔP_i	control	fructose	ΔP_i
ATP	2.60	1.03	-4.71	2.31	1.53	-2.34
ADP	1.22	1.02	-0.40	1.18	1.15	-0.06
AMP	0.40	0.48	+0.08	0.43	0.52	+0.09
Inorganic phosphate	5.3	1.8	-3.50	3.8	2.3	-1.5
P_i uptake (0–4 min)			-0.48			-1.14
Sum			-9.01			-4.95
Fructose 1-phosphate	1.4	8.3	+6.9	0.07	3.67	+3.60
L-glycerol 3-phosphate	0.29	1.47	+1.18	0.29	0.49	+0.20
Sum			+8.08			+3.80
Recovery			90%			78%

rate of oxygen uptake in the period 2-4 min after addition of fructose. III. a 30% increase in the rate of oxygen uptake in the following period. The percent values are given with reference to the prefructose oxygen uptake. In the fasted animal and in the ethanol metabolizing liver of fed animals the oxygen uptake in period II does not decrease below the prefructose level (Hassinen et al. 1970, Zehner et al. 1973, Sestoft 1974 a).

The increase in oxygen uptake in period I seems to be related to an increase in the concentration of ADP which is of very short duration (Raivio et al. 1969). The following sharp decrease in the rate of oxygen uptake is due to a block of the respiratory chain at cytochrome c (Hassinen & Ylikhari 1970) which is also reflected in a concomitant increase in the hydroxybutyrate to acetoacetate concentration ratio (Sestoft 1974 b) and is in equilibrium with the mitochondrial free NADH/NAD . The mechanism of this effect is not clear at present, but seems to be related to an intramitochondrial shortage of inorganic phosphate of very short duration. The effect, which is very similar to the Crabtree effect seen in ascites tumor cells (see Koobs 1972), is not abolished by a high concentration of inorganic phosphate in the medium (Sestoft 1974 a) indicating that the effect is not an overall shortage of phosphate in the hepatocytes. On the other hand the effect is completely released by thyroxine in pharmacological doses which causes an uncoupling of oxydative phosphorylation (quoted from Ylikhari 1970). The effect has been released also in ascites tumor cells

by dinitrophenol (Wu & Racker 1959). As uncoupling releases the dependence of oxygen uptake on the concentration of ADP and inorganic phosphate, this observation seems to indicate shortage of one of the substrates for oxidative phosphorylation during fructose induced 'Crabtree' effect. As the cellular content of ADP is unchanged (Sestoft 1974 b) the candidate must be inorganic phosphate the concentration of which is at its minimum at the same time (Raivio et al. 1969, Hassinen et al. 1970, Sestoft 1974 b).

The persisting increase in the rate of oxygen uptake seen in period III seems not to be related to changes in ADP or in inorganic phosphate the concentrations of which are unchanged compared to a steady state situation without fructose in the perfusion medium (Sestoft 1974 b). On the other hand the effect seems neither to be specific for fructose since addition of various substrates causes an increased rate of oxygen uptake by perfused organs (cf. Lundsgaard 1950). The effect is most clearly shown during fasting in which condition addition of carbohydrate may furnish the Krebs cycle with precursors (oxaloacetate) which are necessary for optimal oxidation of fatty acids. In the case of livers metabolizing fructose the activation of pyruvate kinase by fructose 1-phosphate (Eggleston & Woods 1970) in connection with an activation of pyruvate dehydrogenase caused by the decreasing concentration of ATP (Söling & Bernhard 1971), may lead to an increased influx of carbon into the Krebs cycle. Actually fructose has been shown to increase the concentrations of acetyl-S-CoA (Söling & Willms 1971) and of citrate in man and pig

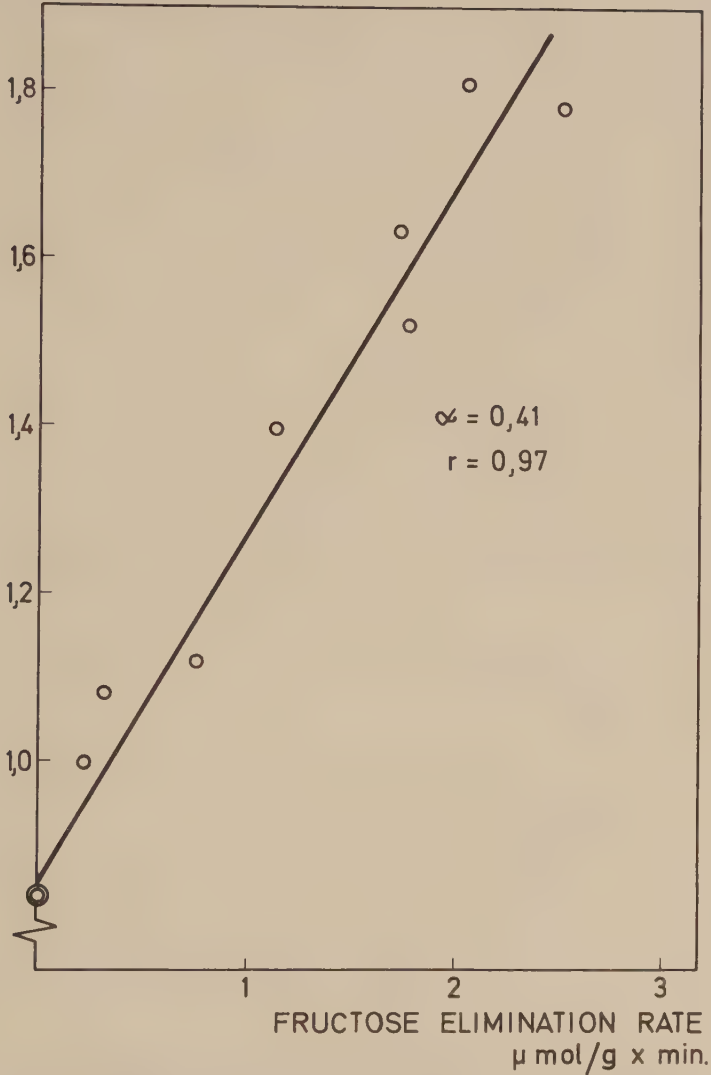
(Miller et al. 1952, Sestoft et al. 1972) and of malate in pig liver (Sestoft et al. 1972). However, the interrelation between a substrate effect on the activity of Krebs cycle and the respiratory control by ADP and inorganic phosphate has not yet been clarified.

The inhibition of the rate of oxygen uptake (Crabtree effect) is accompanied by a sequence of changes in the NAD coupled redox pairs leading to a transient release of reduced substrates (i.e. sorbitol, glycerol and hydroxybutyrate) in the initial 10 min period (Sestoft 1974 b), whereas the rate of glucose release from livers of fasted animals undergoes a small decrease of short duration (Zehner et al. 1973) presumably due to shortage of ATP.

INTERRELATION BETWEEN FRUCTOSE AND ETHANOL METABOLISM.

In man fructose causes an increase in the rate of ethanol oxidation (Stuhlfauth & Neumaier 1951, Lundquist & Wolthers 1958). This effect is due to fructose oxidation in the liver (Tygstrup et al. 1965). In the perfused pig liver the increase in the rate of ethanol oxidation is proportional to the rate of fructose uptake (Damgaard et al. 1973) (Fig. 7). On the other hand the effect cannot be obtained in perfused rat liver (Ylikhari et al. 1971, Sestoft 1974 b). As preparations like liver slices or isolated hepatocytes has a reduced capacity for ethanol oxidation (cf. Thieden & Lundquist 1967, Grunnet et al. 1973) these preparations seem not to be adequate for the investigation of the mechanism of the stimulation of ethanol metabolism in vivo. The perfused pig liver

ETHANOL ELIMINATION RATE
 $\mu\text{mol/g} \times \text{min.}$



L.M. 12-1-72

Fig. 7. The ethanol elimination rate as a function of
the rate of fructose uptake in perfused livers
of fed pigs.

metabolizes ethanol at the same rate as the in vivo pig liver (cf. Damgaard et al. 1974) and we have therefore chosen this preparation for the investigations of the 'fructose effect'. The crucial question is how the liver gets rid of the increased amount of NADH produced during stimulated ethanol oxidation. Investigations with the perfused liver of fed pigs revealed that the rate of oxygen uptake does not account for both the ethanol oxidation to acetate and for the oxidation of the amount of acetate retained in the liver (Damgaard et al. 1972, Damgaard et al. 1973). Therefore release or accumulation of products which are reduced compared to fructose must take place. Glucose has the same redox level as fructose, and the release of sorbitol and glycerol only accounted for a few percent of the required reoxidation of NADH. Therefore accumulation of fatty acids seems to be the only possibility. We have proposed one mechanism by which fructose may facilitate transhydrogenation from NADH produced by ethanol oxidation to NADPH which is coenzyme for the biosynthesis of fatty acids (Thieden et al. 1972) (Fig. 8). The functioning of such a pathway under these special circumstances has not yet been proved, but in the liver of fasted animals, in which no lipogenesis takes place (Wyshak & Chaikoff 1953), the stimulation of ethanol oxidation does not exceed what can be accounted for by oxygen uptake (Damgaard et al. 1974).

If the malic enzyme shuttle is of any significance for the fructose effect, the activity of the malic enzyme should be the rate limiting step for the transhydrogenation (Thieden et al. 1972). Malic enzyme is however readily inducible by

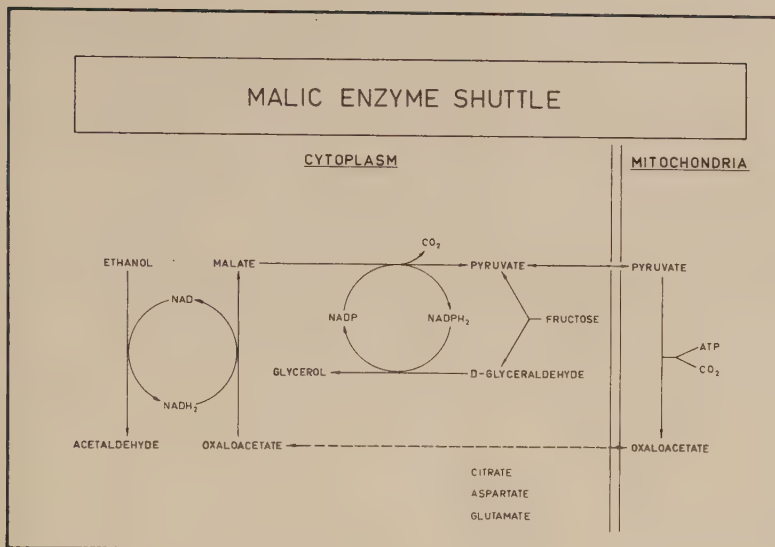


Fig. 8. Metabolic scheme of malic enzyme shuttle.

This figure shows the hypothetical mechanism by which fructose stimulates the ethanol uptake. The mechanism was proposed on the basis of kinetics of rat liver enzymes involved in fructose and ethanol metabolism and experiments with rat liver slices (Thieden et al. 1972). The crucial point is that the rate of ethanol uptake is increased by means of an oxidation of NADH by malate dehydrogenase (oxaloacetate to malate) and the reduction equivalents are then transferred to NADP by malic enzyme (malate to pyruvate). The whole mechanism thus provides a transhydrogenation mechanism by which reduction equivalents generated by ethanol oxidation are transferred to NADPH which is then used for fatty acid synthesis. Thus the NADH produced during ethanol metabolism does not necessarily require reoxidation by means of oxidative phosphorylation. This may explain the experimental results obtained by perfused livers of fed pigs, in which the rate of oxygen uptake did not account for the rate of ethanol oxidation plus oxidation of the acetate retained in the liver (cf. Damgaard et al. 1973).

fructose (Fitch & Chaikoff 1960), and therefore the difference in fructose effect between perfused rat and pig liver may be a consequence of differences in the diets of the animals. Considerations like these open a field of investigations on the reliability of observations done with the perfused isolated organs, and at this point virtually no development has taken place since the days of Lundsgaard (1950).

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